# *A Comparative Microscopic and Biochemical Study of the Uptake of Fluorescent and $^{125}$I-Labeled Lipoproteins by Skin Fibroblasts, Smooth Muscle Cells, and Peritoneal Macrophages in Culture*

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**Uptake of low density lipoprotein (LDL) and of acetyl LDL was compared in skin fibroblasts, smooth muscle cells, and peritoneal macrophages with the use of lipoproteins labeled with either $^{125}$I or the fluorescent probe 3,3'-dioctadecylindocarbocyanine (DiI). The uptake of DiI-labeled lipoproteins was assessed by quantitative spectrofluorometry and by fluorescence microscopy. The DiI was quantitatively retained by the cells, while the $^{125}$I-LDL was degraded and $^{125}$I-labeled degradation products were excreted from the cells. In smooth muscle cells and fibroblasts the uptake of LDL was virtually the same whether measured with the use of the DiI or $^{125}$I-label (sum of cell-associated and degraded $^{125}$I). The labeling of acetyl LDL with DiI enhanced its uptake in peritoneal macrophages by an average of 18%. With the DiI label, lipoprotein uptake (DiI-LDL for smooth muscle cells and skin fibroblasts and DiI-acetyl-LDL for mouse peritoneal macrophages) could be determined after as little as 10 minutes of incubation at 37 C. The pattern of uptake of the DiI-labeled lipoproteins was consistent with binding to specific receptors, because no DiI could be detected in mutant cells without LDL receptors, and uptake was competitively inhibited by addition of excess unlabeled lipoprotein. When the DiI-labeled lipoproteins were removed from the medium, there was a 5–15% loss of DiI from all cell types studied over the first 24 hours. Thereafter, DiI loss from cells was dependent on cell type and culture medium. No further loss of DiI occurred from skin fibroblasts for up to 96 hours of incubation in medium supplemented with either lipoprotein-deficient serum (LPDS) or 10% fetal bovine serum. During this same time period there was a 40–60% loss of DiI from smooth muscle cells and macrophages incubated in medium supplemented with LPDS. Most of the DiI lost from the cells (60–70%) could be recovered in the culture medium but was not the result of cell death, as was indicated by the relatively constant protein concentrations per dish. The loss of DiI was markedly reduced in smooth muscle cells and macrophages when 10% fetal bovine serum was substituted for the LPDS in the culture medium. This suggests that some cells incubated with LPDS undergo changes, perhaps in the plasma membrane, that alter their ability to retain the DiI. In the presence of 10% fetal bovine serum, however, the DiI label is quantitatively retained by all cells tested for up to 96 hours. As a result, DiI provides a potentially useful alternative to radiolabeling for quantification of lipoprotein uptake. The DiI label also permits parallel morphologic evaluation of lipoprotein uptake by fluorescence microscopy, which has several potential applications to the study of the cellular events in atherosclerosis. (Am J Pathol 1985, 121:200–211)**

THE PATHOGENESIS of atherosclerosis is a complex process involving the interaction of a variety of components with the cellular elements of the arterial wall. Central to an understanding of the pathogenesis of this disease is the role of lipoproteins and their metabolism by the cells of the developing atherosclerotic lesion. By the use of a variety of ultrastructural and biochemical techniques, it is clear that the lipid-filled foam cells of the atherosclerotic plaque arise from both smooth muscle cells and macrophages.[1,2] The macrophages appear to have their origin as blood monocytes.[3,4] Little is known about the specific biochemical events that are responsible for foam cell formation in the arterial wall

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and whether the mechanisms are different for smooth muscle cells and macrophages. The development of cell culture techniques has provided a mechanism for studying the metabolism of these cells *in vitro*. With these techniques it has been shown that smooth muscle cells and a number of other cell types possess plasma membrane receptors for low density lipoproteins (LDL). The LDL is taken into the cell by receptor-mediated endocytosis and delivered to the lysosomes, where the LDL is degraded, which makes cholesterol available to the cell. This is known as the LDL receptor pathway (for a review, see Goldstein and Brown[5]). On the other hand, macrophages have very few LDL receptors, and instead possess receptors for the uptake of abnormal lipoproteins. An example is LDL that has been altered chemically by acetylation, which is taken up by what has been termed the "scavenger receptor." Another abnormal lipoprotein, β-VLDL, which is produced in certain cholesterol-fed animals and accumulates in the plasma of patients with Type III hyperlipoproteinemia, is taken up by a different receptor, the β-VLDL receptor (for a review, see Brown and Goldstein[6]). Lipoprotein uptake by the scavenger and β-VLDL receptors results in the accmulation of massive amounts of cholesteryl esters in the macrophage. Because receptors for normal LDL are found on smooth muscle cells but not macrophages and receptors for abnormal lipoproteins are found on macrophages but not smooth muscle cells, this provides a potential mechanism for distinguishing between these cell types in the atherosclerotic lesion. Making this distinction requires the development of a procedure for labeling the lipoproteins in such a way that their uptake by different cells can be discriminated microscopically as well as biochemically.

Recent studies by Pitas and co-workers[7,8] have described a labeling procedure using the fluorescent compound 3,3′-dioctadecylindocarbocyanine (DiI). DiI-labeled acetyl LDL and native LDL have been used to visually identify macrophages, fibroblasts, and smooth muscle cells in both pure and mixed culture systems and in cells derived from arterial explants.[7,8]

The purpose of the present study was to further characterize the use of DiI-labeled lipoproteins for the study of cellular lipoprotein metabolism. The uptake of the DiI-labeled lipoproteins was compared with that of the homologous $^{125}$I-labeled lipoproteins in a variety of cell types for determination of the extent to which the spectrofluorometric determination of cellular fluorescence can be used to quantify LDL uptake and for determination of the extent and conditions under which the DiI is retained by cells in culture. These quantitative measures were compared in parallel experiments with the cellular appearance of the DiI as determined by fluorescence microscopy.

## Materials and Methods

### Lipoprotein Isolation and Labeling

Low density lipoproteins were isolated as previously described from the plasma of rhesus monkeys (*Macaca mulatta*) consuming a cholesterol-containing diet.[9] LDL was labeled with $^{125}$I by the iodine monochloride method as described previously.[9] Labeling of the LDL with the fluorescent probe DiI (Molecular Probes, Inc., Junction City, Ore) was accomplished with the use of a minor modification of the method described by Pitas et al.[8] For this, filter-sterilized (0.45 μ, Gelman Acrodisc) LDL was diluted to a concentration of 1 mg LDL protein/ml in phosphate-buffered saline (PBS).[9] Sufficient lipoprotein-deficient serum (LPDS) was added to give a ratio of LDL protein to LPDS protein of 1:5. The LPDS was prepared from calf serum.[10] A stock solution of DiI was prepared by dissolving 3 mg DiI in 1 ml of dimethyl sulfoxide. The stock DiI was added to the LDL–LPDS mixture to give a final concentration of 150 μg DiI/mg LDL protein. This solution was gently mixed and incubated for 18 hours at 37 C. After the incubation period the density of the mixture was adjusted to 1.080 g/ml with solid KBr and overlayered with 4 ml of 1.063 g/ml KBr solution. The labeled LDL was isolated at 4 C by centrifugation at 36,000 rpm for 24 hours in a Beckman SW-40 rotor. The top 3 ml, containing the DiI-labeled LDL, were collected by tube slicing and dialyzed overnight against 2 liters of 0.9% NaCl and 0.01% ethylenediaminetetraacetic acid (EDTA). The DiI-LDL was sterilized by filtration through a 0.45-μ filter and stored under sterile conditions at 4 C. DiI-LDL was acetylated with acetic anhydride by the method of Goldstein et al.[11]

### Cells

Monkey aortic smooth muscle cells (MSMCs) and skin fibroblasts (MSFs) were obtained from the tunica media of the thoracic aorta and from the skin of normal adult rhesus monkeys as described previously.[10] Normal human skin fibroblasts, skin fibroblasts from a patient with the receptor negative form of familial hypercholesterolemia (GM-2000), and skin fibroblasts from patient J.D. with the internalization-defective form of familial hypercholesterolemia (GM-2408) were obtained from the Human Genetic Mutant Cell Repository, Camden, New Jersey. Resident mouse peritoneal macrophages (PMs) were obtained from 6-week-old female Swiss albino mice (Harlan-Sprague-Dawley, Indianapolis, Ind) by peritoneal lavage essentially as described by Edelson and Cohn.[12]

Both smooth muscle cells and skin fibroblasts were grown as described previously[9] in Eagle's minimal es-

sential medium (Auto-Pow) supplemented with twice the normal concentration of vitamins (Eagle's vitamins), 10% fetal bovine serum (FBS), 23 mM sodium bicarbonate, 200 mM L-glutamine, 1.5 mg α-D(+)-glucose/ml, 100 IU penicillin/ml, and 100 mg streptomycin/ml. This is referred to as growth medium. All cells used in experiments were between the 6th and 22nd passage with a 1:3 split at each passage. For biochemical studies, $1 \times 10^6$ cells were plated into 100-mm tissue culture dishes and for microscopic studies $1.5 \times 10^5$ cells were plated onto tissue culture chamber/slides (#4802, Lab-Tek, Miles Labs, Naperville, Ill). Three days later the growth medium was replaced with "experimental medium" consisting of growth medium in which the fetal bovine serum was replaced by 2.5 mg protein/ml of LPDS. Experiments were initiated after the cells had been incubated in the presence of this LPDS-containing medium for 24–48 hours. Under these conditions the cells were 80–100% confluent in 100-mm dishes and 60–80% confluent in slide chambers.

Peritoneal macrophages were suspended in growth medium without serum and plated onto 35-mm dishes and slide chambers at densities of $6 \times 10^6$ and $3 \times 10^6$ cells per dish or slide chamber, respectively. Sufficient fetal bovine serum was added to each dish or slide chamber to give a final concentration of 20%. Cells were allowed to adhere for 2–15 hours, and nonadherent cells were removed by rinsing several times with growth medium. The adherent cells were cultured in growth medium for 24 hours prior to the addition of the experimental medium containing the lipoproteins to be studied.

All cells were incubated with the various lipoprotein preparations for the times and at the concentrations indicated in the legends to the figures and Table 1.

### Analysis of Lipoprotein Uptake

After the indicated period of incubation, monolayers of smooth muscle cells and skin fibroblasts from 100-mm dishes were washed extensively with PBS containing bovine serum albumin (2 mg/ml) followed by PBS alone as described previously.[9] Cells were detached from the dish with trypsin-EDTA, and the detached cells were washed with three changes of PBS with albumin and two changes of PBS alone. The washed cells were resuspended in 1 ml deionized water, then disrupted for 5 sec. by sonication with a microtip probe at a 30-watt setting of a Heat Systems model 185 sonifier. Macrophages were extensively washed as described above and removed from the dish by scraping with a rubber policeman. The detached cells were disrupted by sonication as described above.

The DiI content of labeled LDL and acetyl LDL in the cell sonicates was determined by measuring the fluorescence of DiI following extraction of the lipoproteins and cells by the method of Bligh-Dyer.[13] The DiI was quantitatively extracted into the chloroform phase and was analyzed for fluorescence in an Aminco-Bowman spectrofluorometer with an excitation of 520 nm and an emission of 578 nm. DiI standards were prepared in chloroform.

For measurement of the uptake of $^{125}$I-LDL the cells were washed and detached from the dishes as described above. The cell-associated LDL was determined after disrupting the cells by sonication. Degraded LDL was determined as trichloroacetic-acid-soluble, noniodide $^{125}$I in the culture medium. Bound LDL in MSMCs and MSFs was determined as the $^{125}$I radioactivity released from the washed cells by trypsin-EDTA treatment. These procedures have been described in detail elsewhere.[9] The protein content of cells and lipoproteins was determined by the method of Lowry as modified by Kashyap et al.[14]

All experiments were repeated at least once with between two and four replicate samples for each data point.

### Microscopy and Photography

Cell monolayers for fluorescence microscopy were incubated with DiI-lipoproteins for the indicated times, rinsed with five changes of PBS with albumin and two changes of PBS, and fixed with cold 3% phosphate-buffered formalin. Coverslips were secured with gelatin mounting medium. Fluorescence microscopy was performed with a Leitz Ortholux fluorescence microscope. Epifluorescent illumination was obtained with a rhodamine filter package. Photographs were made with Kodak Tri-X Panchromatic film.

## Results

The DiI standard curve was linear over a range of 10–1000 ng DiI/ml $CHCl_3$ with 10 ng DiI/ml $CHCl_3$ being the useful lower limit of sensitivity. The DiI concentration of the lipoproteins used in this study was determined by spectrofluorometric analysis on the chloroform extract of each of the lipoprotein preparations. Concentrations were expressed as ng DiI/μg LDL protein. The mean incorporation of DiI into LDL for those preparations used in these experiments was 28.2 ± 6.3 (SD) ng DiI/μg LDL protein (n = 5). The number of molecules of DiI per molecule of LDL was estimated from the mass ratio of DiI (Mr 964) to LDL protein (Mr 695,000–1,285,000). The LDL protein molecular weight was calculated from the molecular weight of the LDL determined as described previously[9] and the per-

cent protein composition (20.7 ± 4.6) of the LDL used in each experiment. On the average, 26 molecules of DiI were present per molecule of DiI-LDL.

Initial experiments in which acetyl LDL was labeled with DiI exactly as described above revealed a marked reduction in the DiI labeling efficiency, compared with that of LDL. The mean incorporation of DiI into these preparations was 5.9 ± 2.0 ng DiI/μg acetyl LDL protein (n=2). This represents approximately 4 molecules of DiI per molecule of acetyl LDL. Because of this low labeling efficiency of acetyl LDL, all DiI-acetyl-LDL used in these experiments was prepared by acetylation of LDL that had been previously labeled with DiI. The acetylation procedure resulted in no significant loss of DiI from the DiI-LDL.

Monkey skin fibroblasts, smooth muscle cells, and mouse peritoneal macrophages were incubated at 37 C with DiI-LDL or DiI-acetyl-LDL at a concentration of 10 μg protein/ml. Cells were incubated for the times indicated in Figure 1 and analyzed for DiI content. The uptake of DiI-LDL by monkey skin fibroblasts and smooth muscle cells was most rapid during the first 30 minutes of incubation; there was a linear but slower rate of uptake over the next 90 minutes. The pattern of uptake of DiI-acetyl-LDL by macrophages was similar, except that on a per-milligram-of-cell-protein basis there was greater uptake of acetyl LDL by the macrophages. In addition, the rate of uptake of acetyl LDL continued to increase between 60 and 120 minutes. This is consistent with results by others showing little down-regulation of the scavenger receptor with accumulation of LDL cholesterol.[15] There was no detectable uptake of DiI-LDL by macrophages and no detectable uptake of DiI-acetyl-LDL by monkey skin fibroblasts or smooth muscle cells during 2-hour incubation (data not shown). The uptake of the DiI-labeled LDL or acetyl LDL markedly inhibited by coincubation with a 20-fold excess (200 μg protein/ml) of the unlabeled homologous lipoprotein (Figure 1). This is consistent with the specificity of uptake by the LDL or acetyl LDL (scavenger) receptor pathways.

The pattern of uptake of labeled lipoproteins by fibroblasts is illustrated in Figure 2, which demonstrates a progressive accumulation of cytoplasmic fluorescence. The fluorescence was most intense in the perinuclear region corresponding to the general distribution of lysosomes (Figure 2B–D). The fluorescent material was occasionally organized in linear arrays, presumably along parallel stress fibers of the cell (Figure 2C), as has been previously described with the use of monoclonal antibodies to LDL receptors[16] and DiI-labeled lipoproteins.[8] The general pattern of accumulation of DiI by smooth muscle cells and macrophages was similar to that of fibroblasts, except that in macrophages the fluorescent

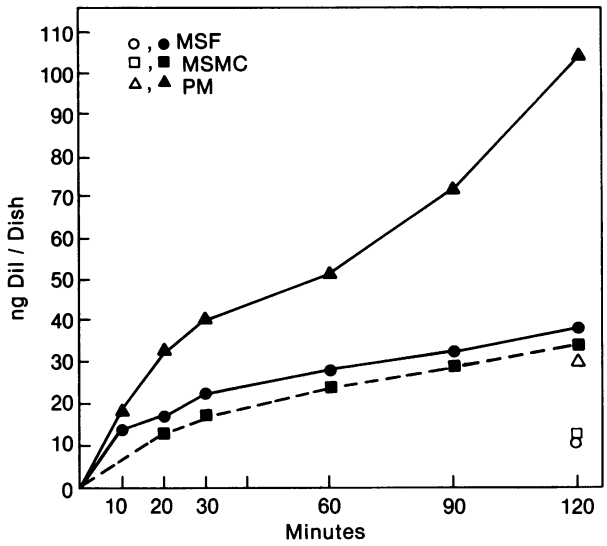


**Figure 1**—Uptake of DiI-lipoproteins by monkey skin fibroblasts (*MSF*), monkey smooth muscle cells (*MSMC*), and mouse peritoneal macrophages (*PM*). MSFs and MSMCs were incubated with 10 μ DiI-LDL protein/ml (●, ■) and PMs with 10 μg DiI-acetyl-LDL protein/ml (▲) at 37 C for the indicated times. Identical incubations were done in the presence of a 20-fold excess of unlabeled LDL (○, □) or acetyl LDL (△). The MSF averaged 0.59 mg protein/dish; the MSMCs, 0.48 mg protein/dish, and the PMs, 0.54 mg protein/dish. Each point represents the mean of duplicate dishes.

material coalesced into large cytoplasmic pools. (Examples of this are illustrated in Figure 6A and C.)

To confirm that the uptake of DiI-LDL was the result of a receptor dependent process, we incubated cultured skin fibroblasts from normal human subjects, patients with an inherited lipoprotein internalization defect (JD), and patients with the LDL receptor negative form of familial hypercholesterolemia (FH) with 10 μg/ml DiI-LDL for 2 hours at 37 C. One group of cells was examined by fluorescence microscopy, and another was analyzed for DiI content by the spectrofluorometric assay. As illustrated in Figure 3, the distribution of fluorescence in the normal human skin fibroblasts was consistent with the uptake of the DiL-LDL by the LDL receptor pathway (panel A). In FH and JD fibroblasts there was considerably less fluorescence. The fluorescence that was present, however, appeared to be localized on the surface of the cell (particularly JD fibroblasts) rather than within the cell. When cellular fluorescence was measured, there were similar concentrations in both normal human and monkey cells, less than 20% of normal concentrations in JD fibroblasts, and no detectable fluorescence in FH fibroblasts (Table 1).

In order to determine the stability of the DiI label in cells, we pulse-labeled skin fibroblasts and smooth muscle cells with DiI-LDL, and macrophages were pulsed-labeled with DiI-acetyl-LDL by incubation at 37 C for 2 hours. After the pulse period, the monolayers

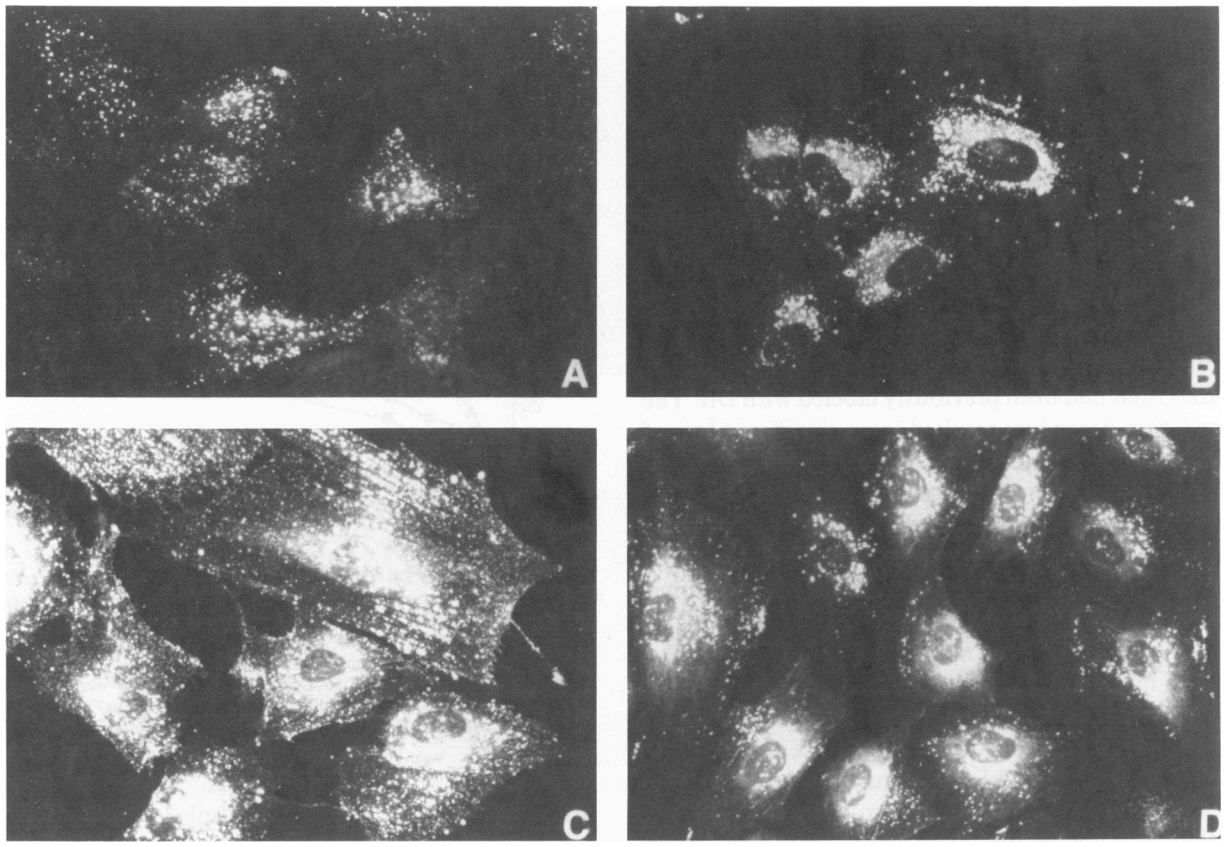


**Figure 2**—Microscopic appearance of DiI fluorescence with duration of incubation by monkey skin fibroblasts. Cells were incubated with 10 μg DiI-LDL protein/ml at 37 C for 10 (**A**), 30 (**B**), 60 (**C**), and 120 (**D**) minutes. After these periods of incubation the cells were washed, fixed, and prepared for fluorescence microscopy as described in Materials and Methods. (×810)

were washed extensively as described in Materials and Methods and incubated with fresh medium supplemented with either LPDS or 10% fetal bovine serum during the chase period of the experiment. Cells were harvested at 24-hour intervals up to 96 hours and analyzed for DiI and protein content (Figure 4).

The amount of cell protein per dish remained constant or increased slightly throughout the 96-hour incubation in medium containing LPDS. This is in contrast to the consistent increase in protein per dish when maintained on serum-containing medium. This probably represents some cell growth in the case of fibroblasts and smooth muscle cells. In macrophages the increase in cell protein appeared to be due to the growth of nonmacrophage, fibroblastlike cells. Others have reported the appearance of fibroblastlike cells among cultured peritoneal exudate cells.[17] As a result of this increase in cell protein per dish, the amount of DiI retained during the 96-hour chase period was expressed on a per-dish basis. After the 2-hour pulse period the absolute amount of DiI in the cells was similar to that shown in Figure 1. Within the first 24 hours of the chase period there was a 5–15% decrease in the amount of DiI per dish for all of the cell types. During the subsequent 72 hours there was little additional loss of DiI from fibroblasts regardless of medium composition. Smooth muscle cells and macrophages, however, lost 40–50% of their DiI by 96 hours when cultured in medium supplemented with LPDS. This did not appear to be the result of cell death, because the amount of cell protein per dish did not decrease. The loss of DiI was markedly reduced when the cells were incubated in the presence of fetal bovine serum during the chase period.

In order to determine whether the DiI was lost from the cell or altered into a nonfluorescent form, we extracted the LPDS medium from the 48-, 72-, and 96-hour chase intervals after a 24-hour pulse period and assayed the chloroform extract for DiI using the described spectrofluorometric procedure. In smooth muscle cells 67% of the DiI lost from the cells during the chase period was recovered from the culture medium, and in macrophages 62% was recovered. In control studies using an identical extraction procedure, 76%

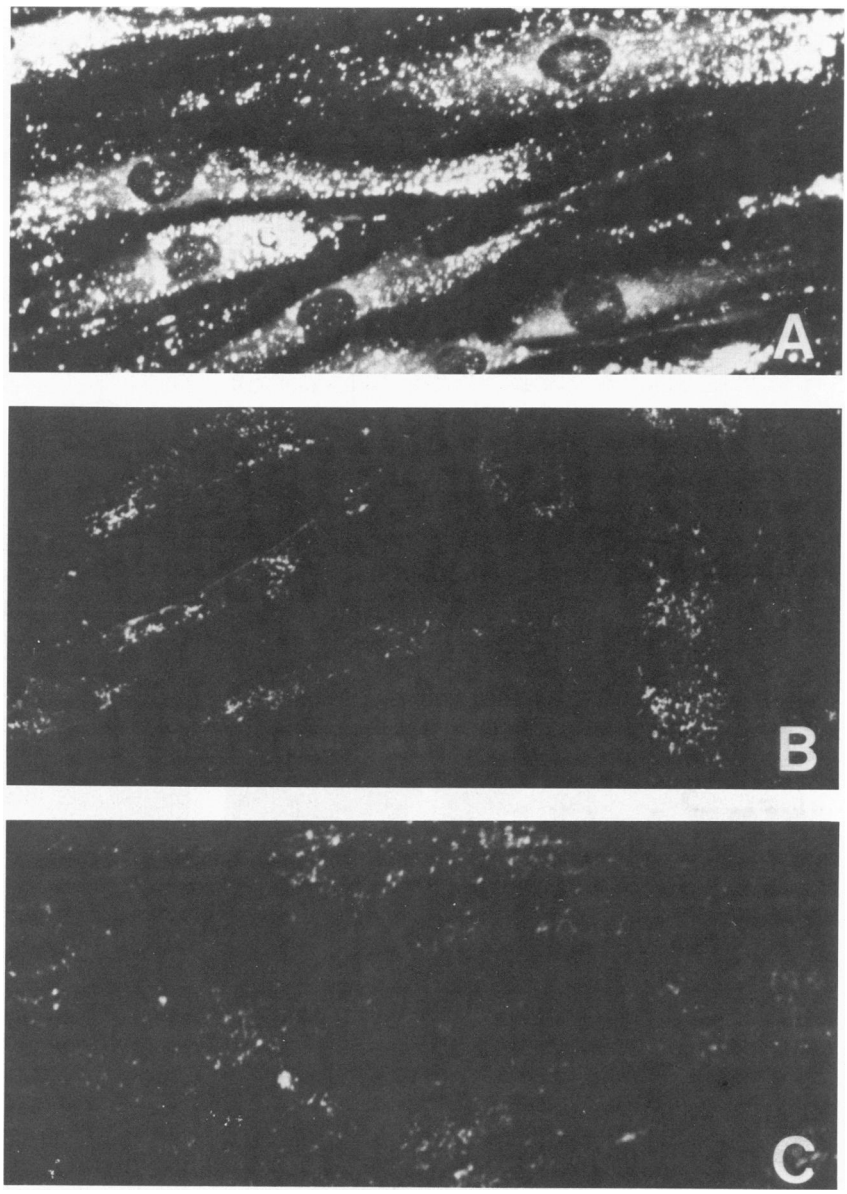


**Figure 3**—Microscopic appearance of cellular DiI fluorescence after incubation of normal human skin fibroblasts (**A**), fibroblasts from patient J.D. with the LDL internalization defect (**B**), and fibroblasts from a patient with the LDL-receptor–negative form of familial hypercholesterolemia (**C**). Cells were incubated with 10 μg protein/ml of DiI-LDL for 2 hours at 37 C, washed, fixed, and prepared for fluorescence microscopy as described in Materials and Methods. (×900).

of a known quantity of DiI dissolved in methanol and added to LPDS medium in culture dishes with or without cells was recovered. Similar experiments were not done with the chase medium from skin fibroblasts because there was minimal loss of DiI from these cells when incubated with LPDS medium.

The cellular appearance of the DiI fluorescence in skin fibroblasts during the chase period is illustrated in Figure 5. At the end of the 2-hour pulse labeling period, the bulk of the fluorescence was concentrated in the perinuclear region of the cell with some diffuse fluorescence detectable throughout the cytoplasm (Figure 5A). After the 24-hour chase period there was redistribution of the cellular fluorescence into intensely

Table 1—Uptake of DiI-LDL by Normal and Mutant Skin Fibroblasts

| | ng DiI/dish |
|---|---|
| Normal monkey | 29 |
| Normal human | 32 |
| Internalization defective (JD) | 6* |
| Familial hypercholesterolemia (FH) | 0 |

Cells were incubated with 10 μg protein/ml of DiI-LDL at 37 C for 2 hours in the presence and absence of a 20-fold excess (200 mg protein/ml) of unlabeled LDL. DiI uptake in the presence of the 20-fold excess of unlabeled LDL ranged from 10 to 11 ng/dish. This value was subtracted from the cellular DiI content in the absence of the 20-fold excess of LDL so that the specific uptake of DiI could be determined. Results are the mean of duplicate determinations.

* This value is below the level of reliability of the spectrofluorometric assay for DiI and should be considered as an estimate only.

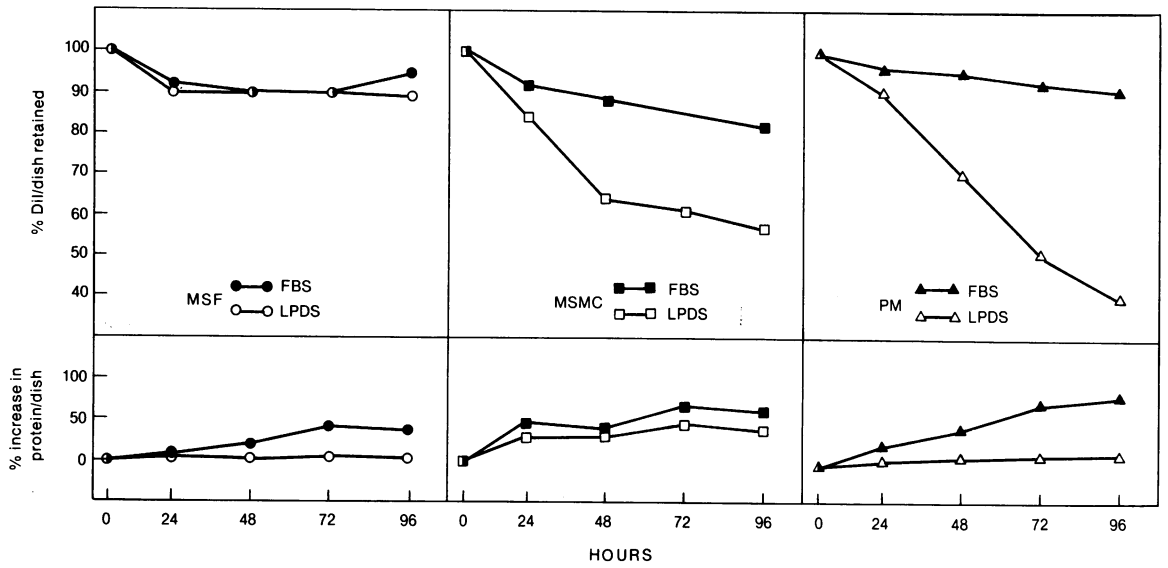


**Figure 4**—Retention of cellular DiI in medium containing either lipoprotein-deficient serum (*LPDS*) or fetal bovine serum (FBS). Monkey skin fibroblasts (*MSF*), (○, ●), monkey smooth muscle cells (*MSMC*) (□, ■), and mouse peritoneal macrophages (*PM*) (△, ▲) were incubated with DiI-LDL (MSFs, MSMCs) or DiI-acetyl-LDL (PMs) (10 μg protein/ml) for 2 hours at 37 C (pulse period) in 35-mm dishes for the purpose of loading the cells with DiI. Cells were then washed with PBS, one group was harvested at 0 time, and the remaining cells were incubated for the indicated time in medium containing either LPDS (open symbols) or FBS (closed symbols). Triplicate dishes were pooled at each time period, and cells were assayed for DiI and protein content. The DiI content of the cells at 0 time was 12, 10, and 35 ng/35-mm dish for MSFs, MSMCs, and PMs, respectively. The 0 time protein content for the same dishes was 0.20, 0.15, and 0.18 mg/35-mm dish, respectively. The data point for MSMCs at 72 hours in FBS was lost.

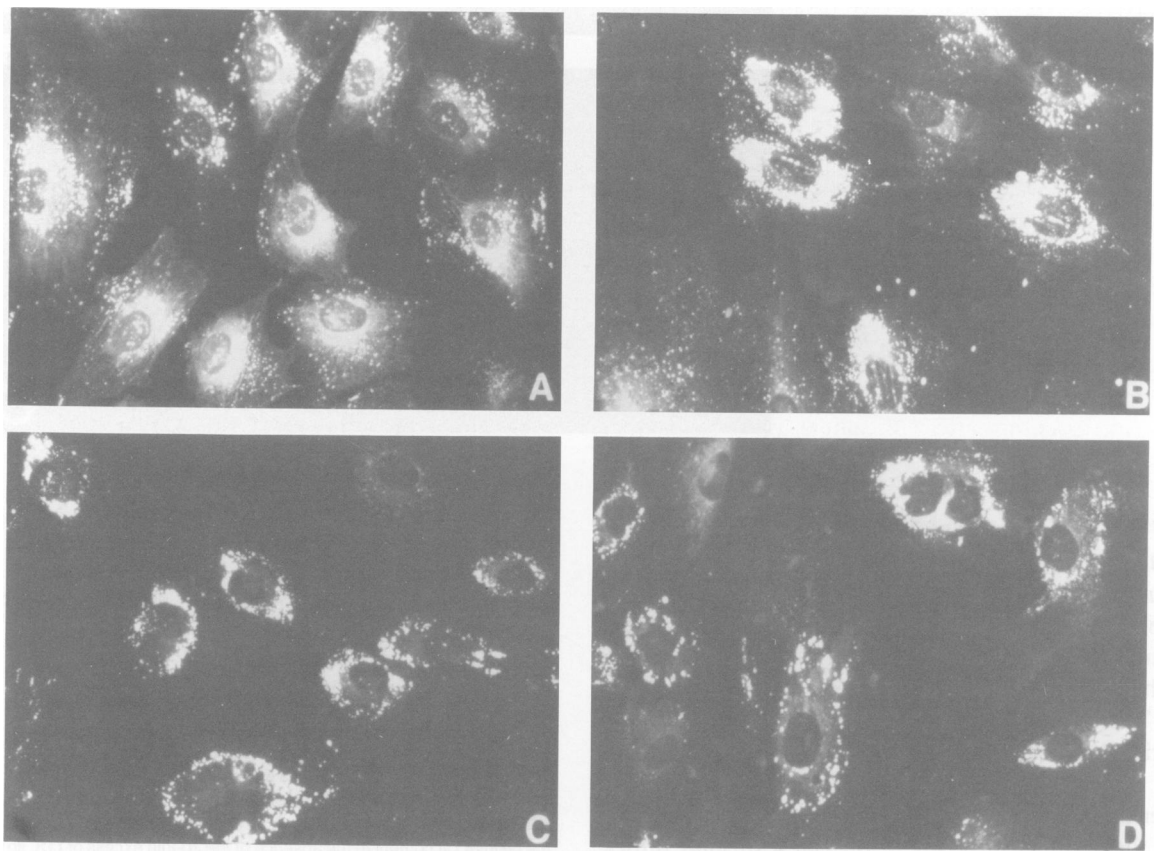


**Figure 5**—Microscopic appearance of cellular DiI fluorescence in monkey skin fibroblasts after incubation in lipoprotein-free medium. Cells were incubated for 2 hours at 37 C (pulse period) with DiI-LDL as described for Figure 3. Cellular fluorescence after the pulse period is shown in **A.** Cells were then incubated at 37 C for up to 96 hours in medium containing LPDS exactly as described in the legend to Figure 3. Cellular fluorescence was examined after 24 (**B**), 48 (**C**), and 72 (**D**) hours in cells washed, fixed, and prepared for fluorescence microscopy as described in Materials and Methods. (×810)

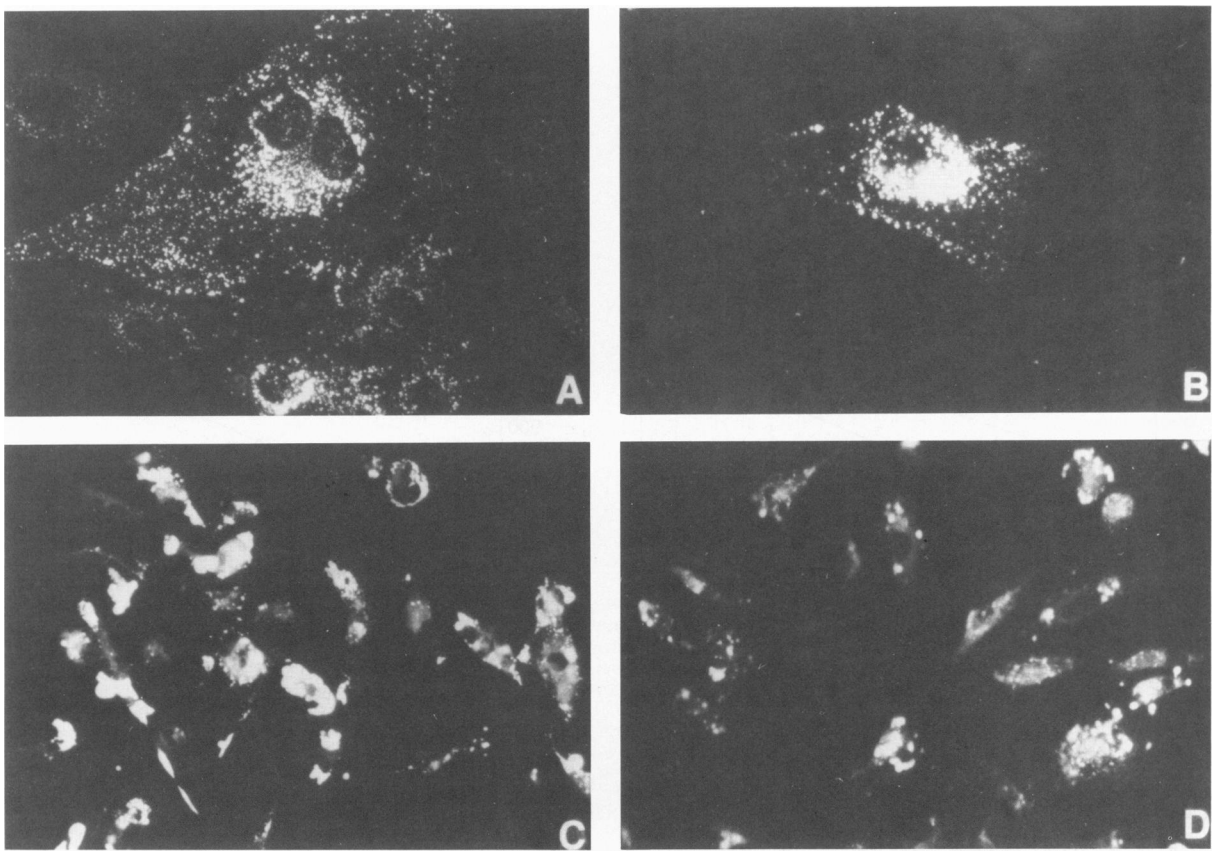


**Figure 6**—Microscopic appearance of cellular DiI fluorescence in monkey smooth muscle cells and mouse peritoneal macrophages after incubation in lipoprotein-free medium. MSMCs and PMs were incubated for 2 hours at 37 C (pulse period) with DiI-LDL and DiI-acetyl-LDL, respectively, as described in Figure 3. Cellular fluorescence after the pulse period is shown in **A** (MSMCs) and **C** (PMs). Cells were incubated and processed as described in the legend to Figure 4, and cellular fluorescence after a 72-hour chase period is illustrated in **B** (MSMCs) and **D** (PMs). (×810)

fluorescent perinuclear regions (Figure 5B). After 48 and 72 hours there appeared to be further coalescence and some redistribution of the fluorescent material to the periphery of the cell (Figure 5C–D). Although some similarities in the pattern of cellular fluorescence were seen for the smooth muscle cells and macrophages (Figure 6B and D), the diffuse cytoplasmic fluorescence and redistribution of fluorescence was much less pronounced in these cells. The decrease in the cellular content of fluorescent material, as determined microscopically, was most apparent in the macrophages (Figure 6C–D).

In order to determine whether uptake of LDL could be accurately quantified by measurement of DiI fluorescence of the cells, we incubated identically prepared dishes of skin fibroblasts, smooth muscle cells, and macrophages at 37 C with experimental medium containing 5 μg $^{125}$I-LDL protein/ml and 5 μg DiI-LDL protein/ml (fibroblasts and smooth muscle cells) or 5 μg $^{125}$I-acetyl-LDL protein/ml and 5 μg DiI-acetyl-LDL protein/ml (macrophages). At each time point the nanograms of $^{125}$I-lipoprotein internalized and degraded were calculated from the specific activity of the $^{125}$I-lipoprotein. In addition, the ng of $^{125}$I-LDL bound to the cell surface was determined for fibroblasts and smooth muscle cells from the amount of $^{125}$I released after treatment of the cells with trypsin. In a similar manner, the uptake of DiI-lipoprotein was determined by dividing the nanograms of DiI per dish by the specific activity of the DiI-lipoprotein. It was not possible to determine lipoprotein bound with the use of the DiI fluorescence, because the amount of bound fluorescence was below the lower limit of sensitivity. The results of this experiment are shown in Figure 7. It is apparent from these data that the pattern of uptake of the $^{125}$I and DiI-labeled lipoproteins is remarkably similar.

To determine the extent to which the two methods of labeling lipoproteins would give similar quantitative values for total lipoprotein metabolism, we compared the sum of $^{125}$I-lipoprotein internalized and degraded at each time point with cellular DiI uptake. The $^{125}$I bound was not included, because surface-bound LDL is lost and would not be quantified after trypsin treat-

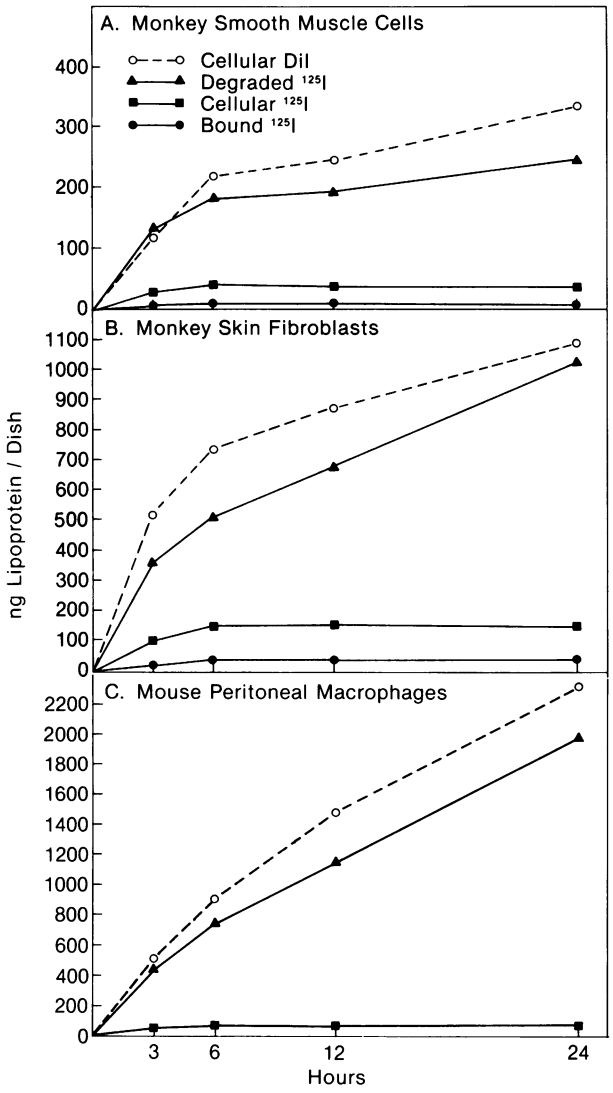


**Figure 7**—Comparison of cellular uptake of DiI-lipoproteins with binding, internalization, and degradation of $^{125}$I-lipoproteins. Cells were incubated at 37 C with 5 μg protein/ml of DiI-lipoprotein and 5 μg protein/ml of $^{125}$I-lipoprotein. At the indicated times the cells were harvested and analyzed for either DiI content or $^{125}$I-lipoprotein bound (as determined by trypsin release), $^{125}$I cell-associated and $^{125}$I degraded. Results are the mean of duplicate dishes. The monkey smooth muscle cells averaged 0.39 mg protein/dish; the monkey skin fibroblasts, 0.80 my protein/dish; and the mouse peritoneal macrophages, 0.41 mg protein/dish.

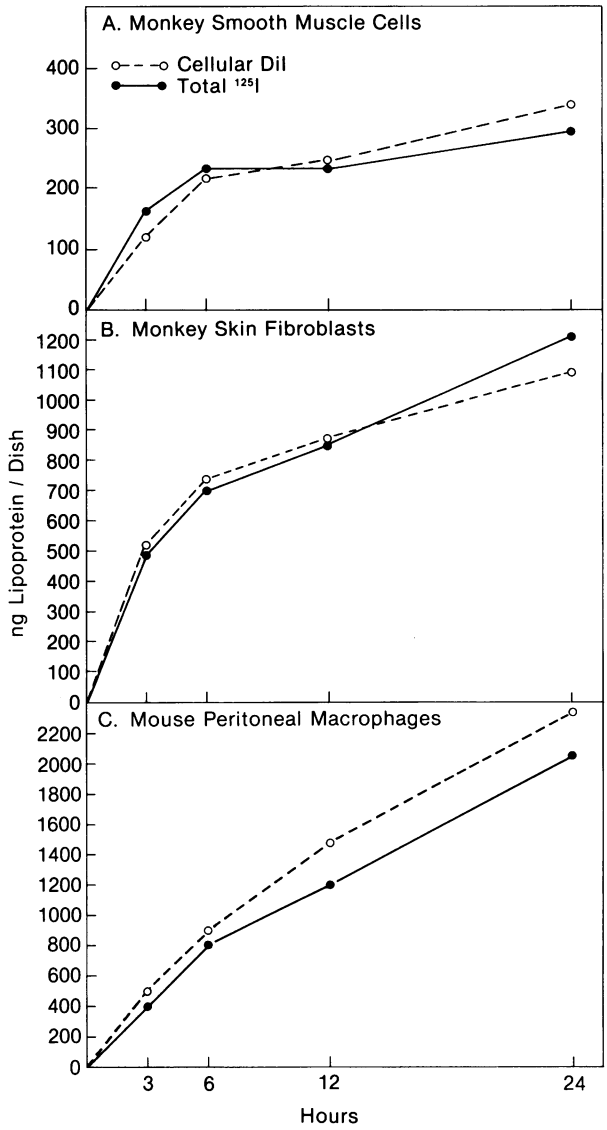


**Figure 8**—Comparison of cellular uptake of DiI-lipoproteins with total $^{125}$I-lipoproteins metabolized. The data from Figure 7 have been compared by representing the sum of $^{125}$I cell-associated and degraded as the total $^{125}$I-lipoprotein metabolized and comparing this value with the cell associated DiI. Results are the mean of duplicate dishes.

ment of the cells incubated with DiI-LDL. Surface-bound LDL, however, represents only a small percentage (<5%) of total $^{125}$I-LDL metabolized under these conditions. As shown in Figure 8, DiI-labeled LDL gave quantitative values for the uptake of LDL by both skin fibroblasts and smooth muscle cells that were essentially identical with that of $^{125}$I-LDL. However, approximately 18% more DiI-acetyl-LDL than $^{125}$I-acetyl-LDL was taken up by peritoneal macrophages.

To determine whether this greater uptake of DiI-acetyl LDL was truly the result of the DiI label, rather than some unrecognized error in the quantification of the uptake of DiI-LDL, we incubated mouse peritoneal macrophages with DiI-$^{125}$I-acetyl-LDL or $^{125}$I-acetyl-LDL that was prepared from a single batch of $^{125}$I-acetyl-LDL. The effect of the DiI label on the uptake and degradation of the $^{125}$I-acetyl-LDL is shown in Figure 9. Consistent with the results in Figure 8, the addition of the DiI produced a 20% increase in the uptake and degradation of acetyl-LDL.

## Discussion

Results of this study have confirmed and extended previous reports by others[7,8,18-20] using DiI-labeled

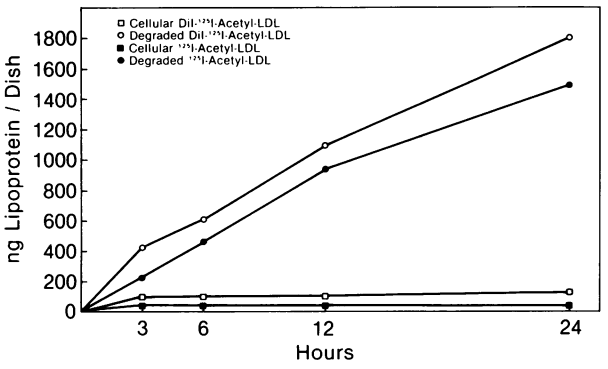


**Figure 9**—Comparison of internalization and degradation of $^{125}$I-acetyl-LDL (■, ●) and DiI-$^{125}$I-acetyl-LDL (□, ○). Mouse peritoneal macrophages were incubated at 37 C with 10 μg protein/ml of either $^{125}$I-acetyl-LDL or DiI-$^{125}$I-acetyl-LDL. A single batch of $^{125}$I-acetyl-LDL was prepared, and a portion was taken for labeling with DiI. At the indicated times the cells were harvested and analyzed for $^{125}$I cell-associated and $^{125}$I degraded. Results are the mean of triplicate dishes. The cells averaged 0.46 mg protein/dish.

lipoproteins to follow the uptake of lipoproteins by skin fibroblasts, smooth muscle cells, hepatocytes, and macrophages in culture. Under appropriate conditions the DiI from uptake of DiI-labeled lipoproteins is quantitatively retained by cells, which makes it possible to use the DiI label to quantify the uptake of LDL by the spectrofluorometric determination of DiI in lipid extracts from the cells. The use of the DiI label to quantify lipoprotein uptake has been validated by comparison with the uptake and degradation of $^{125}$I-lipoproteins. Another advantage of this technique is that the same DiI-labeled lipoproteins can be used for the microscopic examination of cellular uptake. Thus, both quantitative uptake and morphologic characteristics can be evaluated in parallel.

The sensitivity of the spectrofluorometric method depends on two principal factors: 1) the limit of detectability of the DiI, which is approximately 10 ng/ml chloroform under the conditions of this study, and 2) the extent of DiI labeling of the lipoproteins. In the current study there were approximately 26 molecules of DiI per molecule of LDL (28.2 ng DiI/μg LDL protein). This compares with approximately 45 DiI molecules per LDL molecule reported in the studies by Barak and Webb[19] and approximately 55 ng DiI/μg LDL protein in the studies by Pitas et al.[8] These differences in the degree of labeling are probably attributable to a number of factors, including minor differences in the methodology of labeling, differences in the source of lipoproteins, and differences in the composition of the lipoproteins. Increasing the number of DiI molecules per molecule of LDL, within the range used by us and others, does not appear to affect the chemical, physical, or biologic properties of the LDL[8,19]; however, data from this study (Figure 9) suggests that the presence of DiI on acetyl LDL enhances its uptake by macrophages. The extent to which this is affected by the degree of labeling with DiI was not tested. This effect of DiI on uptake of acetyl LDL by the macrophage scavenger receptor is not entirely unexpected. A variety of chemical and biologic alterations in LDL will enhance uptake by the scavenger receptor.[6] One potential mechanism whereby DiI could alter the uptake of acetyl LDL is by a change in the surface charge of the acetyl LDL due to the net positive charge of each DiI molecule. It is well known that alterations in charge of the protein can have major affects on binding to the scavenger receptor.[6] Regardless of the mechanism, the addition of a similar number of DiI molecules to LDL had little influence on uptake via the LDL receptor pathway and did not induce the uptake of DiI-LDL by macrophages in these experiments.

A consistent finding of the current study was the reduced efficiency of DiI labeling of acetyl LDL (5.9 ng DiI/μg lipoprotein), compared with DiI-LDL, which was subsequently acetylated (28.2 ng DiI/μg lipoprotein). The DiI is thought to associate with the surface of the lipoprotein in much the same manner as phospholipid.[19] Therefore, the reduced DiI labeling of the acetyl LDL may be the result of steric hindrance by the acetyl groups for access of DiI to the lipoprotein surface. Another possibility is that the acetylation alters the surface polarity of the LDL thus affecting the partitioning of DiI at the LDL surface during the labeling procedure. As a result, if a high degree of labeling is desired, it is better to label the LDL with DiI before acetylation.

Net uptake of DiI-LDL could be detected spectrofluorometrically in normal skin fibroblasts and smooth muscle cells after approximately 10 minutes of incubation at 37 C. When compared with the uptake of $^{125}$I-LDL (sum of cell associated + degraded $^{125}$I), DiI-LDL uptake was virtually identical at all time points studied up to 24 hours of incubation. This suggests that there is little loss of DiI from the cells during the 24 hours that DiI-LDL is in the culture medium.

When the DiI-LDL was removed from the medium, the cellular content of DiI decreased by 5–15% in the first 24 hours, regardless of the cell type or composition of culture medium. In skin fibroblasts there was no further loss of DiI for up to 96 hours of incubation, regardless of the culture medium. In contrast, smooth muscle cells lost up to 40% of their DiI content by 96 hours when cultured in medium containing lipoprotein-deficient serum. This loss was reduced to 18% in the presence of whole serum. A similar pattern was observed in macrophages, with the 60% loss at 96 hours in lipoprotein-deficient serum-containing medium being reduced to a 9% loss when cultured in the presence

of whole serum. Much of the DiI lost from smooth muscle cells and macrophages during the chase period could be recovered from the culture medium, suggesting that the DiI was lost from the cells rather than being converted into a nonfluorescent molecule. The effect of whole serum in preventing the loss of DiI is presumably due to the presence of lipoproteins, because lipoprotein-deficient serum contains all of the plasma proteins except the lipoproteins. The initial 5–15% loss of DiI occurred in all cells during the chase period, regardless of media composition, which suggests that it may represent a different mechanism than occurred during longer incubations with LPDS only. One possibility is the retroendocytosis of newly internalized lipoproteins.[21] The long-term retention of DiI by cells incubated in the presence of serum observed in this study is similar to the results reported by Pitas et al,[7] who showed that in neither human skin fibroblasts nor mouse peritoneal macrophages was there loss of DiI after incubation for up to 120 hours in medium supplemented with serum. The loss of DiI from macrophages and skin fibroblasts incubated in LPDS medium cannot be attributed to cell death, because the amount of cell protein per dish remained constant or increased slightly. The most likely possibility for the loss of DiI in the absence of whole serum is that serum lipoproteins are necessary for the maintenance of normal cell membrane function and in their absence cell membrane turnover is enhanced and cellular integrity is abnormal, resulting in the loss of DiI. If this is true, then there must be some difference in membrane structure or metabolism in skin fibroblasts, compared with smooth muscle cells and macrophages. The difference in DiI retention by the various cell lines could also be related to the pattern of cytoplasmic redistribution of DiI seen during the chase phase in fibroblasts, but to a lesser extent in smooth muscle cells and macrophages. This peripheral redistribution may represent either active or passive transfer of the DiI from the perinuclear region of cells (presumably within lysosomes) to other cellular locations. Further characterization of this phenomenon and the effects of lipoprotein-deficient serum on the retention of DiI might provide new insights into the role of lipoproteins in such cellular processes as lysosomal stability and membrane turnover.

Pitas et al[7,8] have shown that DiI-labeled LDL and acetoacetylated LDL can be used to distinguish smooth muscle cells from macrophages in mixed cell cultures and in cells that migrate from atherosclerotic plaques onto tissue culture dishes. Although theoretically this approach could be used for the determination of the relative number of foam cells derived from macrophages and smooth muscle cells within an atheroma, this technique has not yet been successfully applied to atherosclerotic tissues. Because specific uptake of DiI-LDL occurs only in cells with LDL receptors (smooth muscle cells and skin fibroblasts) and acetoacetylated LDL and acetyl LDL are taken up only by macrophages and perhaps also endothelial cells[22] that posses scavenger receptors, it may be possible by incubating atherosclerotic lesions with DiI-labeled lipoproteins to quantify spectrofluorometrically the accumulation of DiI from specific DiI-labeled lipoproteins and thus estimate the relative proportion of macrophages and smooth muscle cells. Furthermore, the DiI label provides a simple method for labeling lipoproteins (perhaps under conditions where radiolabeling cannot be used) and for simultaneous visualization and quantification of their uptake by cells in culture.

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